IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Patent Application of

SUSAN E. BARRIE *ET AL.*		Atty. Ref:	**604-291**
Serial No.	**08/315,882**	Group:	**1202**
Filed:	**September 30, 1994**	Examiner:	**Rizzo, N.**

For: **17-SUBSTITUTED STEROIDS USEFUL IN CANCER TREATMENT**

#9
11-15-95

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RULE 132 DECLARATION

SUSAN ELAINE BARRIE declares as follows:

1. I am one of the inventors of the above application, residing at "Fosterbrook", 1 Park Cottages, Westerham, Kent TN16 1SJ, England. I am employed by the Institute of Cancer Research at their laboratories in Belmont, Sutton, Surrey, England, in the post of Research Scientist and have had 18 years experience in the field of biological testing of anti-cancer agents.

2. I am aware that there is some prior art, considered pertinent to this patent application, relating to certain cardiotonic steroids which differ from the compounds of the above patent application. One of the differences is that the compounds of the prior art are unsaturated in the 16 - and 17 - positions of the steroid D - ring, the 3 - pyridyl group being in the 17β-orientation, whereas the compounds of the above application have a 16,17-double bond and only one orientation of the 3-pyridyl group at the 17-position is therefore possible.

3.. I tested the ability of a number of compounds of the invention of the above patent application and other compounds to inhibit the steroid 17α-hydroxylase-$C_{17,20}$-lyase enzyme, obtained from the human testis.

This enzyme is responsible for androgenic hormone biosynthesis which produces

dehydroepiandrosterone and androstenedione, immediate precursors of testosterone, from their respective precursors pregnenolone and progesterone, in both testes and adrenals. It is well recognised that shutting off the supply of androgens to the prostatic cells is a useful step in therapy of prostatic cancer. The ability of a test compound to inhibit the enzyme is therefore a significant indicator of its efficacy as an anti-prostatic cancer agent. Such tests are indeed reported in the specification of the above patent application.

4. The procedure used was similar to that described in the specification of the above patent application, but as there were some minor variations it is convenient to set it out in full below.

5. Enzyme Preparation and Assay Procedure for the 17*a*-Hydroxylase-$C_{17,20}$-lyase Enzyme. The ^{3}H-labelled compounds were obtained from NEN Products, Stevenage, Herts, U.K. The biochemical reagents were from Boehringer Mannheim, U.K., Lewes, East Sussex, U.K., or Sigma Chemical Co. Ltd., Poole, Dorset, U.K. The chemicals were of analytical grade.

A microsomal fraction was prepared by the method of Chasalow, J. Biol. Chem.1979, *254*, 3000-3005, from human testes removed at orchiectomy from previously untreated patients with cancer of the prostate. The microsomes were resuspended in 50 mM sodium phosphate buffer (pH. 7.4)-glycerol (3:1) at the equivalent of 1 mL/g of fresh tissue and stored in liquid nitrogen until use.

The assay was based on that of Chasalow, Steroids, 1982, *30,* 617-630, and the assay mixture contained 3 μM ^{3}H-labelled substrate (1-3μCi/nmol) 250 μM NADPH, 10mM D-glucose 6-phosphate, 1mM $MgCl_2$, 2U/mL D-glucose 6-phosphate dehydrogenase, 0.1mM $MgCl_2$, 0.1 mM dithiothreitol, 0.2 mM EDTA, 1% ethanol, 1% DMSO, 3% glycerol, and 95% mM sodium phosphate buffer (pH 7.4). The test compounds were prepared in 50% DMSO, the controls receiving just 50% DMSO. The control reaction was carried out at 37°C. It was started by the addition of the microsomal preparation and stopped by the addition of 2 volumes of MeCN-MeOH (1:2) containing unlabelled steroids (ca. 100μM). The samples were stored at -20°C until analysis. The control reaction was linear with time, and the rate was proportional to the protein concentration under the conditions used (data not shown).

HPLC Analysis. (a) Hydroxylase Activity. For measurement of the hydroxylase activity, the substrate was progesterone and the unlabelled steroids added at the end of the assay were progesterone, 17α-hydroxyprogesterone, androstenedione, testosterone, and 16α-hydroxyprogesterone. The samples were injected onto a 10 cm Nucleosil 5-μm C18 column fitted with an Uptight guard column filled with Nucleosil C18 packing. The mobile phase was 60% MeOH at a flow rate of 1mL min^{-1}. The effluent was monitored at 240 nm before being mixed with Ecoscint A containing 25% MeCN and monitored for ^3H using a Berthold LB506C detector. Activity was measured as the production of 17α-hydroxy-progesterone. No androstenedione nor testosterone were produced until the substrate was depleted, and the reaction was not carried out long enough for this to occur.

(b) **Lyase Activity.** For the measurement of the $C_{17,20}$-lyase activity, the substrate was 17α-hydroxyprogesterone and the unlabelled steroids added at the end of the assay were 17α-hydroxyprogesterone, androstenedione, and testosterone. The samples were injected onto a 10 cm Apex 5μM C18 column fitted with an Uptight guard column filled with PELL ODS packing. The mobile phase was H_20-MeCN-MeOH (4:1:3) at a flow rate of 1mL min^{-1}. The effluent was monitored at 240 nm before being mixed with Ecoscint A containing 5% MeCN, 5% MeOH and monitored for ^3H using a Berthold LB506C detector. Activity was measured as the production of androstenedione and testosterone.

Inhibitory Activity: For ease of dissolution, test compounds were first converted into their hydrochlorides. In a typical procedure, HC1 gas was passed through a solution of the base in Et_2O and the hydrochloride which precipitated was recovered by filtration and desiccated. Each compound was tested at a minimum of four different concentrations, and the data were fitted by nonlinear regression to the median effect equation of Chou, J. Theor. Biol. *39*, 253-276 (1976):

$$f_a/f_u=(I/IC_{50})^n$$

where f_a= the fraction of activity affected, f_u= the fraction of activity unaffected, I= the concentration of inhibitor, IC_{50}=inhibitor concentration giving 50% inhibition, and n depends on the sigmoid shape of the curve (n=1 for systems obeying Michaelis-Menten kinetics). The correlation coefficients were all greater than 0.96. This method of analysis was chosen as it is valid for calculating IC_{50} values whatever the IC_{50}: enzyme concentration ratio. In contrast,

methods based on the Michaelis-Menten equation become invalid for values of IC_{50} of less than 100x (enzyme concentration).

The estimates of the enzyme concentration were obtained by fitting some of the data to the equation derived by Henderson, Biochem. J., *135*, 101-107, (1973), for tight binding inhibitors. For a tight binding inhibitor:

$$IC_{50}=K_{i_{app}} +0.5x \text{ (enzyme concentration).}$$

6. Some of the tests which I carried out are germane to the difference between 17-pyridyl -16,17-unsaturated steroids and their 17β-pyridyl-16,17-saturated counterparts. Among the compounds tested were the following (1) to (4) which were prepared by my co-inventor Dr Gerry Potter:

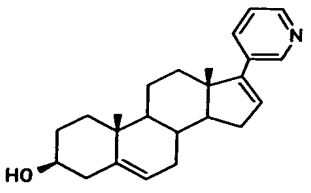
(1): 17-(3-Pyridyl)androsta-5,16-dien-3β-ol

(2): 17β-(3-Pyridyl)androst-5-en-3β-ol

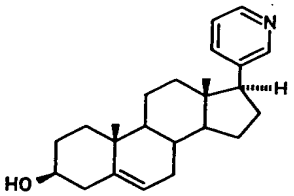
(3): 17-(4-Pyridyl)androsta-5,16-dien-3β-ol

(4): 17β-(4-Pyridyl)androst-5-en-3β-ol

The formulae of these compounds are shown below. It will be observed that (1) is a compound of the above patent application, (2) is its 16,17-saturated-17β-pyridyl counterpart, (3) is the same as (1) except that the pyridyl ring nitrogen is in the 4-position and is therefore not a compound of the above patent application and (4) is the 16,17-saturated-17β-pyridyl counterpart of (3).



(1)



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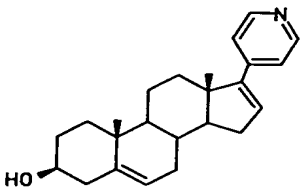

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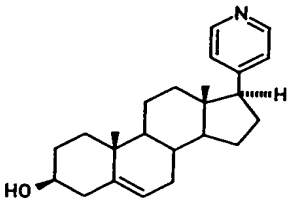

(4)

7. The results of my tests on these four compounds were as follows:

Compound	IC_{50}(nM)[a]	
	$C_{17\text{-}20}$Lyase	17α-Hydroxylase
1	2.9	4
2	23	47
3	1000	4000
4	53	160

a. The standard errors were usually less than 10% of the IC_{50} value. The concentration of enzyme was estimated to be about 4-5 nM.

These results indicate to me that the spatial position of the nitrogen atom in the pyridyl ring relative to the whole steroid molecule is highly critical. When it is in the 3-position of the pyridine ring, as in compounds (1) and (2), the orientation of the pyridine ring in the plane of the steroidal D-ring is clearly highly critical to inhibitory potency, as it was reduced (i.e. inhibition increased) by an order of about a ten-fold when the pyridyl ring was shifted out of the plane of the steroid D-ring by adopting the axial 17β-configuration. On the other hand, the reverse effect was seen in the comparison of (3) with (4). However, despite this improvement, the 17β-(4-

pyridyl) steroid (4) was still not as good an inhibitor of the enzyme under test as the corresponding 17β-(3-pyridyl) steroid (2). My results therefore support the proposition that both the 3-pyridyl situation of the N-atom and the 16,17-double bond are critical features of the invention of the above patent application.

8. I declare further that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true and further that these statements were made with the knowledge that wilful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of title 18 of the United States code and that such wilful false statements may jeopardize the validity of the instant patent application or any patent issuing thereon.

11 July 1995

Date

S.E. Barrie.

Susan Elaine Barrie